AD_____

AWARD NUMBER DAMD17-96-C-6065

TITLE: Characterization and Modulation of Proteins Involved in Sulfur Mustard Vesication

PRINCIPAL INVESTIGATOR: Dean S. Rosenthal, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20007

REPORT DATE: September 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1998	Annual (15 Sep 97 - 14 Sep 98)

4. TITLE AND SUBTITLE
Characterization and Modulation of Proteins Involved in Sulfur Mustard Vesication

5. FUNDING NUMBERS
DAMD17-96-C-6065

6. AUTHOR(S)
Dean S. Rosenthal, Ph.D

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington DC 20007

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Material Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Sulfur mustard (SM) causes blisters in the skin through a series of cellular changes that we are beginning to identify. In the last year, we found a major role for Ca^{2+} and calmodulin in the induction of differentiation in human keratinocytes in response to SM. We also obtained the unexpected results that SM induces markers of apoptosis, and that this process also proceeds via a Ca^{2+}-calmodulin-dependent pathway. In addition, we found that SM-induced apoptosis was also mediated by a FADD-dependent pathway which induces caspase activation. The involvement of such varied molecules as Ca^{2+}, calmodulin, and FADD suggests a complex network involved in SM-induced differentiation and apoptosis. However, in our progress to date, we have found that blocking any one of these upstream signals can inhibit terminal differentiation or apoptosis, indicating that these molecular pathways are potential targets for therapeutic intervention. We will continue to modulate the expression and activation of calmodulin and FADD, and thus the differentiation and apoptotic pathways in cultured keratinocytes and grafted epidermis to alter SM toxicity. In addition, we will establish lines of transgenic mice that express antisense to CAM or a dominant-negative inhibitor of the FADD pathway in order to further study their roles in SM vesication in intact animals.

14. SUBJECT TERMS
Sulfur Mustard; Calmodulin; Differentiation; Apoptosis; Keratinocytes; Caspases

15. NUMBER OF PAGES
22

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

(✓) Where copyrighted material is quoted, permission has been obtained to use such material.

(✓) Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

(✓) Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

(✓) In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 32 CFR 219 and 45 CFR 46.

(✓) In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Principal Investigator's Signature

9/15/98
Date

TABLE OF CONTENTS

1. OVERVIEW

SM is a highly reactive compound that induces the death and detachment of the basal cells of the epidermis from the basal lamina (Gross et al., 1988; Meier et al., 1984; Papirmeister et al., 1991; Petrali et al., 1990; Smith et al., 1990; Smith et al., 1991; Smulson, 1990). During the first year, in collaboration with Drs. William Smith and Radharaman Ray at USAMRICD, I examined whether markers of differentiation and apoptosis are induced or altered by **SM**. I found that **SM** in fact induced both the terminal differentiation response as well as an apoptotic response in keratinocytes (Rosenthal et al., 1998; Stöppler et al., 1998). Utilizing both chemical inhibitors and antisense oligonucleotides, we further determined that Ca^{2+} and calmodulin are important to both responses, and that these two related processes may in part play a role in **SM** toxicity. During the second year we have made a number of significant advances towards the understanding of the mechanisms involved in the response to **SM**. First, we characterized the molecular ordering of events responsible for **SM**-induced apoptosis. Second, we found a Fas/TNF receptor pathway for **SM**-induced apoptosis, and directly tested this pathway by using a dominant-negative inhibitor of FADD. Third, we have continued to generate calmodulin antisense constructs and have packaged these constructs into retroviral vectors and used them to infect primary and immortalized cells. Finally, we have performed grafting experiments utilizing these calmodulin and FADD constructs to examine the role of these proteins in the vesication response to **SM**.

2. SULFUR MUSTARD INDUCES MARKERS OF TERMINAL DIFFERENTIATION AND APOPTOSIS IN KERATINOCYTES VIA A Ca^{2+}-CALMODULIN AND FAS/TNF-DEPENDENT PATHWAY: Further study of the apoptotic pathway.

2.1 Introduction

In the first year we focused primarily on caspase-3 in the apoptotic response. In order to further understand the apoptotic response, we have devoted much of our effort to assay for the activation of other key caspases, in particular the "upstream" caspases 8, 9, and 10, and the "executioner" caspases 3, 6, and 7 (Figure 1), since a number of studies for the past several years have shown that the central signaling proteins for many of the pathways that coordinate apoptosis are members of this family of cysteine proteases (named for their preference for aspartate at their substrate cleavage site (Alnemri et al., 1996). Caspases cleave key proteins involved in the structure and integrity of the cell.

Accordingly, during the past year, I have focused on the roles of Ca^{2+} /calmodulin in the modulation of differentiation and apoptosis in epidermal cells, and potentially involved in vesication, as well as the role of the Fas/TNF receptor pathway. I have utilized much of the same technology that I have successfully employed in the first year to answer an essential question- How do Ca^{2+} /calmodulin and the Fas/TNF receptor family alter the apoptotic and differentiation responses in keratinocytes, and can these pathways be modulated to alter **SM** vesication in animal models (and ultimately, in humans)?

In the first year, we showed that **SM** induces both terminal differentiation and apoptosis in human keratinocytes. Further, we have demonstrated that these processes are Ca^{2+} and calmodulin dependent, and involve the activation of caspase-3. These responses may, in part, explain the death and detachment of basal cells of the epidermis that occurs following exposure to **SM**. Additionally, utilizing a combination of techniques including the stable expression of a dominant-negative inhibitor of **FADD**, we have begun to shown the potential role of a Fas/TNF family receptor in mediating the response of human keratinocytes to **SM (Figure 1)**. Importantly, our initial inhibition experiments indicate that the Ca^{2+}-calmodulin and FADD pathways converge upstream of caspase-3 processing, **since inhibitors of either pathway inhibit SM-induced apoptosis**. Furthermore, since calmodulin inhibitors have been used clinically, and the FADD pathway can be manipulated at the level of a cell surface (Fas/TNF), receptor, these two molecules represent attractive targets for the modulation of the effects of **SM** in humans. Accordingly, during the second year, I focused primarily on the role of Ca^{2+}, calmodulin and FADD in the (premature) induction of differentiation and apoptosis in epidermal cells leading to vesication.

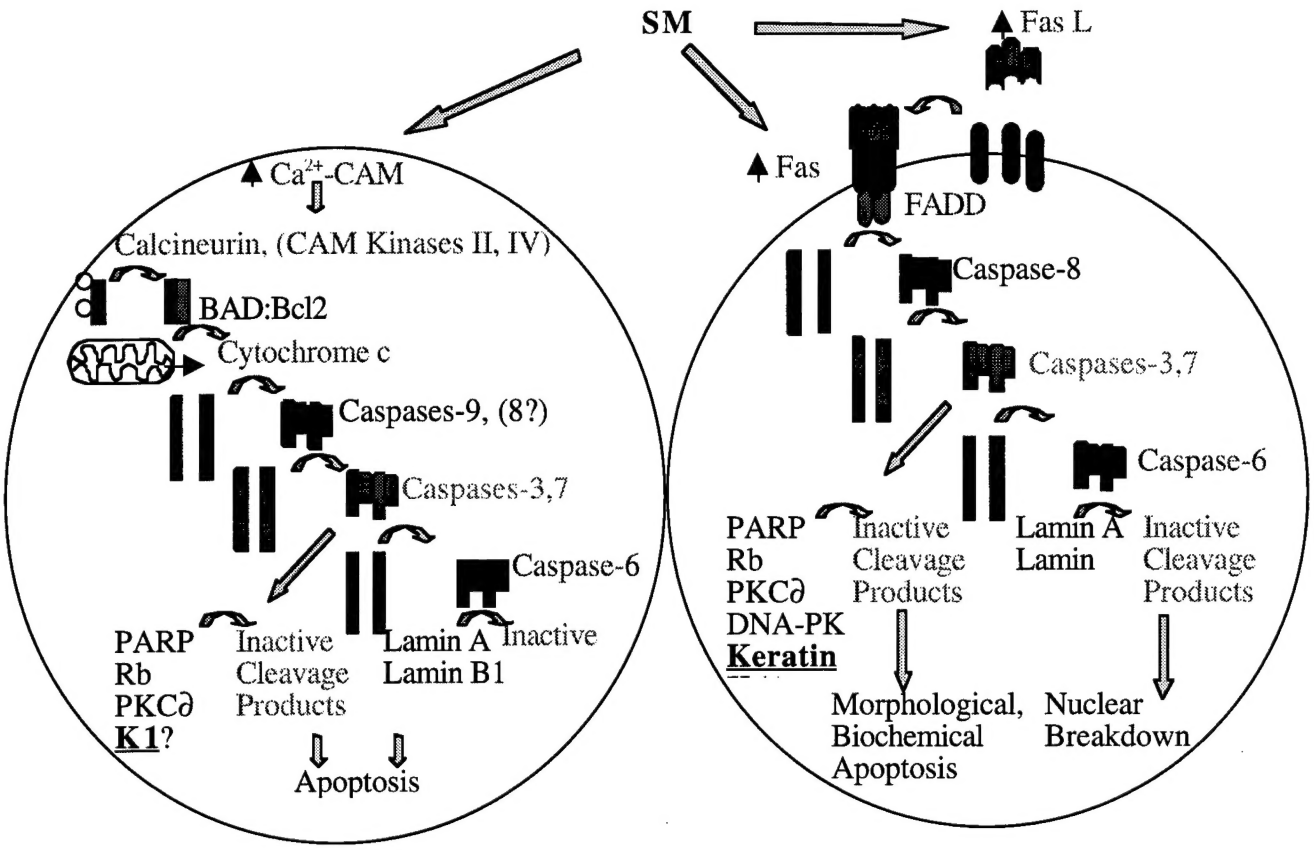


FIGURE 1: Two related pathways for SM-induced apoptosis

2.2 Materials and Methods

(1) ***Culture of primary and immortalized human keratinocytes, and exposure to SM.***

Cells. Normal human epidermal keratinocytes (**NHEK**) are obtained as primary cultures from Clonetics (San Diego, CA) and maintained in serum-free Keratinocyte Growth Medium (KGM). **Nco** are a kind gift from R. Schlegel, and are derived from NHEK immortalized with the Nco I fragment of HPV 18 containing the coding regions for E6 and E7 (Schlegel et al., 1988). NcoIs are grown in KGM + DMEM (3:1) medium containing 2.5% FCS. Cells are grown to 80% confluency and split 1:5. NHEKs or NcoIs are grown in 75 cm^2 tissue culture flasks to 60-80% confluency, then exposed to HD diluted in KGM to final concentrations of 10, 100, 200, or 300 μM. Media is not changed for the duration of the experiments

Chemicals. **SM** (bis-(2-chloroethyl) sulfide; >98% purity) is obtained from the US Army Edgewood Research, Development and Engineering Center.

(2) ***Measurement of proteolytic activation of caspase-2, -3, -6, -7, -8, -9, and -10.***

Since one of the primary goals is to determine the molecular ordering of events leading to **SM**-induced apoptosis, we have employed two different assays for the analysis of the time of onset of activation of each of the caspases. We have extensive experience utilizing Western analysis to detect the activation of several caspases. We have tested a number of antibodies from commercial and collaborative sources for their sensitivities and specificities using cells treated with known apoptosis-inducing agents, such as anti-Fas antibody, as controls. We presently have excellent antibodies specific for all relevant caspases (see antibody Table). In addition, we have antisera that detect the substrate cleavage products for caspases 3 and 7 (several different PARP antisera: Dr. Smulson has a complimentary grant from the Army that focuses on the role of PARP), as well as for lamin B1, a substrate of caspase-6. Thus, by performing time-course experiments, as well as inhibitor studies outlined, we will be able to determine the sequence of events, as well as the regulatory molecules (such as Bcl-2), involved in **SM**-induced apoptosis.

a. ***Fluorogenic caspase enzyme assays.*** In addition to Western analysis to detect caspase activation, we have been employing fluorogenic enzyme cleavage assays utilizing different substrates to assay for specific caspases. The substrates that are utilized are as follows:

Caspase	Specificity	Source
2	ZVDVAD-AFC	Kamiya (Frankfurt, Germany)
3,7	AcDEVD-AMC	Bachem (Bubendorf, Switzerland)
6	AcVEID-AMC	California Peptide Research (Napa, CA)
8	AcIETD-AFC	Kamiya (see above)
4,5,9	AcLEHD-AFC	Kamiya (see above)

These assays are rapid, sensitive, and also verify the results of Western analysis:

Enzyme reactions are performed in 96-well plates and contained 20 µg of cytosolic proteins, in 100 µl of Buffer A, diluted with 100 µl of fresh Buffer A containing 40 µM acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (DEVD-AMC), or other substrates (see Table above). Fluorescent aminomethyl coumarin AMC product formation is measured at excitation 360 nm, emission 460 nm wavelengths using a Cytofluor II fluorometer plate reader (PerSeptive Biosystems, Framingham, MA). Serial dilutions of AMC (Aldrich, Milwaukee, WI) are used as standards.

b. ***Immunoblot analysis.*** SDS-polyacrylamide gel electrophoresis and protein transfer to nitrocellulose membranes are performed according to standard procedures. Membranes are stained with Ponceau S (0.1%) to confirm equal loading and transfer. After blocking of nonspecific sites, the blots are incubated with monoclonal or polyclonal antibodies (above) and then detected with appropriate peroxidase-labeled secondary antibodies (1:3000 dilution) and enhanced chemiluminescence (ECL, Amersham). Immunoblots are sequentially stripped by incubation for 30 min at 50 °C with a solution containing 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7), blocked again, and reprobed with additional antibodies to accurately compare different proteins from the same filter.

c. ***Antibodies.*** All antibodies indicated below have already been tested and used successfully in our laboratory.

Antibody (kDa)	type (clone)	Source	Dilution (conc.)
Calmodulin (17)	monoclonal (6D4)	Sigma (St. Louis, MO)	1:1000
K1 (67)	polyclonal	Babco (Richmond, CA)	1:50
K1; K10 (67; 59)	monoclonal (8.60)	Sigma (see above)	1:100 (1 µg/ml)
K14 (50)	monoclonal	Sigma (see above)	1:200
Involucrin (68)	monoclonal (SY5)	Sigma (see above)	1:200
Fibronectin (220; 94)	polyclonal	Sigma (see above)	1:500
Fas (48)	monoclonal	Transduction Labs (Lexington, KY)	1:250 (1 µg/ml)
Fas ligand	polyclonal	Santa Cruz Biotech(Santa Cruz, CA)	1:400 (0.5 µg/ml)
FADD (24)	monoclonal (1)	Transduction Labs (see above)	1:250
AU1	monoclonal (AU1)	Babco (see above)	1:1000 (1 µg/ml)
Caspase-3 (32; 17)	polyclonal	Dr. D. Nicholson(Merck Labs, Can)	1:5000
Caspase-3 (propeptide)	polyclonal	Transduction Labs (see above)	1:500
Caspase-6 (34; 11)	monoclonal(B93-4)	PharMingen (San Diego, CA)	1:250 (2 µg/ml)
Caspase-7 (35;17)	monoclonal	PhaMingen (see above)	1:500 (1 µg/ml)
Caspase-7 (17)	polyclonal	Dr. E. Gelmann(Georgetown Univ.)	1:1000
Caspase-8 (20)	polyclonal	Dr. E. Gelmann (see above)	1:1000
Caspase-8 (55)	monoclonal	PharMingen (see above)	1:100 (1 µg/ml)
Caspase-9	monoclonal	PharMingen (see above)	1:400
Caspase 10 (55)	polyclonal	PharMingen (see above)	1:500 (1 µg/ml)
PARP (116, 89)	monoclonal (c210)	BioMol (Plymouth Meeting, PA)	1:5000
PARP DBD (24)	polyclonal	Dr. I. Hussein (Res. Triangle, NC)	1:400
PAR	polyclonal	Dr. M. Smulson;D. Rosenthal (GU)	1:500
Lamin B1	monoclonal	Calbiochem (La Jolla, CA)	1:100 (1 µg/ml)
Rb (110)	monoclonal	Calbiochem (see above)	1:100 (1 µg/ml)
DFF45 (45; 30)	polyclonal	PharMingen (see above)	1:1000
p53 (53)	monoclonal(ab421)	Calbiochem (see above)	1:200 (0.5 µg/ml)
Bcl-2 (25)	monoclonal (4D-7)	Biomol (see above)	1:200 (1 µg/ml)

Bcl-X_L	polyclonal	Calbiochem (see above)	1:40 (2.5 μg/ml)
Bax (21)	polyclonal	Calbiochem (see above)	1:50 (2 μg/ml)
Apop. endonuclease	polyclonal	Dr. Yoshihara(Nara Med. U, Japan)	1:1000

(3) ***Hoechst and propidium iodide staining for apoptotic morphology*:** Observation of changes in nuclear morphology (development of apoptotic nuclear morphology: chromatin condensation, nuclear fragmentation). Keratinocytes are isolated by exposure to trypsin followed by resuspension in serum-containing media. The cells are centrifuged (800*g* for 5 min), washed with PBS, and fixed in 10% formalin for 10 min at 4^0C. After washing twice with phosphate-buffered saline (PBS), the cells are stained either with Hoechst 33258 (24 μg/ml) in PBS containing 80% (v/v) glycerol, or with propidium iodide (according to manufacturers specifications) in PBS. An aliquot (25 μl) of the cell suspension is then dropped onto a slide, and nuclear morphology is observed with an Olympus BH2 fluorescence microscope.

(4) ***DNA extraction for detection of apoptotic internucleosomal DNA fragmentation.*** Cells are washed in PBS and lysed in 7 M guanidine hydrochloride, and total genomic DNA is extracted and purified using a Wizard Miniprep DNA Purification Resin (Promega). After RNase A treatment (20 μg/ml) of the DNA samples for 30 min, apoptotic internucleosomal DNA fragmentation is detected by gel electrophoresis on a 1% agarose gel and ethidium bromide staining (0.5 μg/ml). Using this protocol we have repeatedly detected DNA ladders in keratinocytes treated with **SM**.

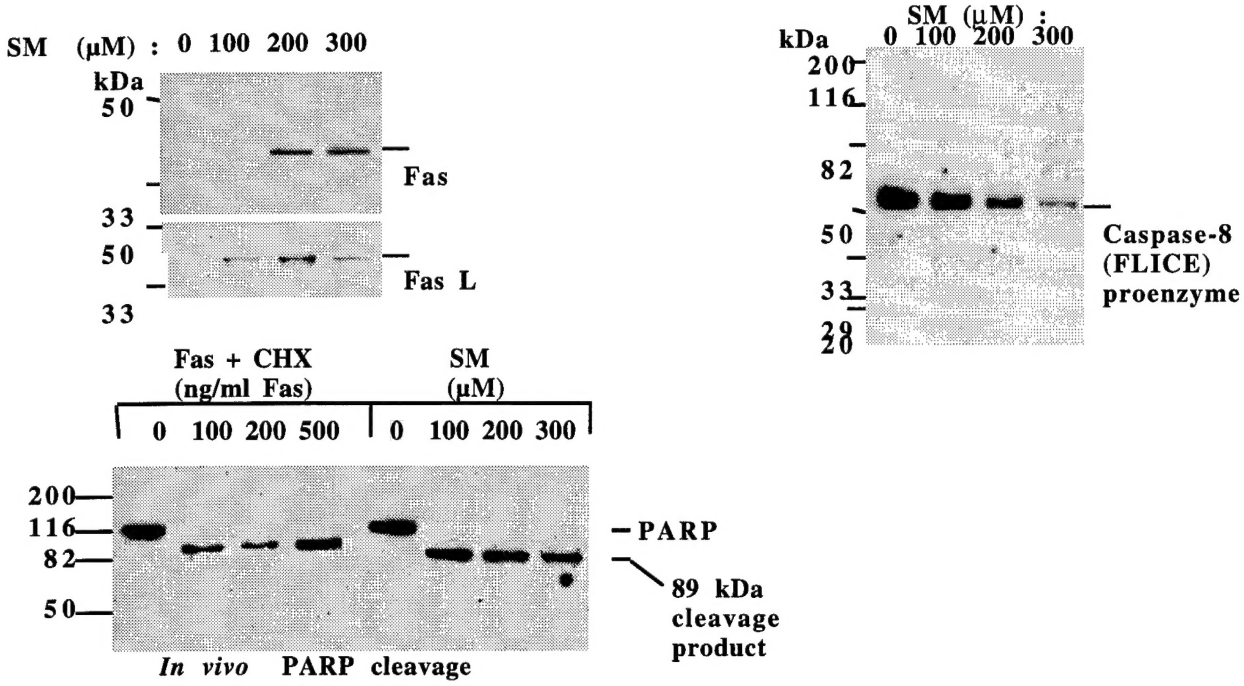
(5) ***Annexin V-binding and FACS analysis.***
Methods to monitor apoptosis-related cell surface changes **Annexin-V and propidium iodide staining.** Keratinocytes are harvested by exposure to trypsin and centrifugation, washed with ice-cold PBS, and stained for 15 min in the dark at room temperature with propidium iodide and fluorescein isothiocyanate-labeled annexin-V (both, according to the manufacturer's specifications) (Trevigen) in a solution containing 10X binding buffer and water. The cells are then examined with a fluorescence microscope, or subjected to FACS analysis, utilizing a FACStar flow cytometer.

(6) ***LDH assays.*** Levels of cell toxicity are assessed by measuring the release of the cytosolic enzyme lactate dehydrogenase into the medium with the use of a Cytotox 96 kit (Promega).

2.3 Results

We first analyzed changes in the expression of apoptotic markers following exposure of primary keratinocytes to **SM**. Consistent with our previous results, we observed a strong activation of caspase-3 activity (detected by the PARP-cleavage assay, the fluorogenic assay, and immunoblot analysis as described in Materials and Methods. In addition to the activation of caspase-3, we tested whether other caspases within the cascade were similarly activated. We found that there was an early activation of caspase-8 and a later activation of caspases- 3, 6, and 7. Since this is similar to the pattern of caspase activation following stimulation of the Fas receptor with either Fas ligand or with agonist antibody, we reasoned that **SM** may in fact induce apoptosis via the Fas pathway, or a related receptor within the Fas/TNF receptor family. First, we subjected primary keratinocytes to treatment with an anti-Fas agonist antibody to induce "pure" apoptosis, and compared the response to that of keratinocytes that were treated with **SM**. The response of the keratinocytes to each of these agents was remarkably similar with respect to the time course and the order of activation of different caspases (**see figure below**).

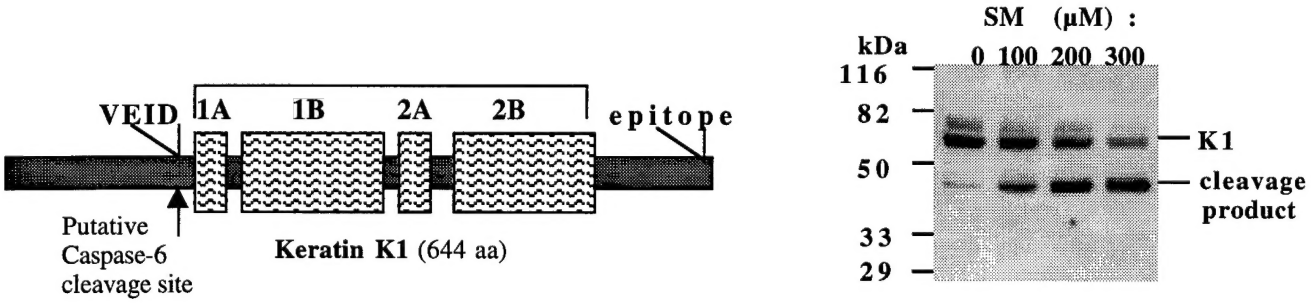
Changes in endogenous levels of apoptosis-mediating receptors Fas and Fas-ligand (Fas-L), as well as caspases and "death domain" signaling intermediates (FADD) during SM-induced apoptosis.



Recent evidence has suggested that certain agents, in particular anticancer drugs, induce apoptosis by up-regulating the levels of Fas receptor, Fas ligand, or both, and experiments have shown that over-expression of either of these proteins can induce apoptosis. In addition, it has been shown that Fas transcription can be up-regulated by p53, which we have shown is rapidly induced in keratinocytes following treatment with **SM**. Thus, we examined the levels of Fas and Fas-ligand following treatment of primary keratinocytes with **SM** utillizing immunoblot analysis employing the antibodies and techniques described above. **The figure above** shows that both Fas and Fas ligand are induced in primary keratinocytes following exposure to **SM**.

Immunoblot and pulse labeling/immunoprecipitation analysis of <u>newly discovered</u> caspase-mediated cleavage of epidermal keratins during SM-induced differentiation and apoptosis.

In order to study the differentiation response to **SM**, we originally focused on the suprabasal-specific keratins, K1 and K10, which are tightly regulated at the level of transcription in keratinocytes both *in vitro* and *in vivo*. **However, many changes may occur at the post-translational level, including a putative caspase-mediated breakdown of keratin K1 that occurs during apoptosis**. Thus, we continued to employ Western analysis, as well as immunofluorescent analysis to examine the changes in these gene products in the second year.



To determine if **SM** alters keratin expression, we performed a time course, followed by Western analysis using a monoclonal antibody specific for the suprabasal keratins K1 and K10, which are the major proteins expressed in keratinocytes in response to differentiating agents. Both K1 and K10 were weakly induced by 10 μM **SM**, and strongly induced in the presence of 100 μM **SM**, between 8 and 24 h following treatment. When we recently used a different polyclonal antibody directed against the C-terminus of K1, and treated cells with higher concentrations of **SM**, we discovered an apparent cleavage product of K1. The size of this product maps near a perfect consensus sequence for a site of cleavage by caspase-6 (**see figure above**). Moreover, point mutations near this region of K1 give rise to a genetic **blistering disorder,** epidermolytic hyperkeratosis (McLean et al., 1994). Thus, it is of interest to determine if K1 can be cleaved by caspase-6 following treatment with **SM**. We are performing the same experiments in the presence of the caspase-6 inhibitor, VEID-CHO. Inhibition of the cleavage product in the presence of the inhibitor would strongly suggest that K1 is in fact the substrate for caspase-6, and that K1 is a target during keratinocyte apoptosis. In order to determine if the smaller molecular weight fragment arises from K1 cleavage, a pulse-labeling experiment will be performed:

Examination of changes in members of the anti-apoptotic Bcl-2 family to examine their role in Ca^{2+} /calmodulin mediated pathways.

Bcl-2 is an anti-apoptotic protein located on the nuclear membrane, ER, and outer mitochondrial membrane. Several Bcl-2-related proteins have been described, including Bcl-XL, and Bcl-w, both of which are anti-apoptotic. In addition, some Bcl-2-related proteins are anti-apoptotic, including Bax, Bak, and Bad. In numerous recent studies, Bad has been implicated as a key player in programmed cell death (Datta et al., 1997; del Peso et al., 1997; Hsu et al., 1997; Scheid and Duronio, 1998; Yang et al., 1995; Zha et al., 1997; Zundel and Giaccia, 1998) and the Ca^{2+} /calmodulin-regulated protein, calcineurin, has been shown to interact with the Bcl-2 family members (Shibasaki et al., 1997), and to dephosphorylate Bad (Wang et al., 1997). This dephosphorylated form of Bad can interact with Bcl-2 or Bcl-X_L and induce apoptosis (Zha et al., 1997).
NHEKs were treated with 100 μM **SM** for 24 h, and then subjected to Western analysis, using a Bcl-2-specific antibody. The figure below shows the significant decrease in the expression of Bcl-2 protein levels following **SM** treatment. p53 has been shown to antagonize this activity of Bcl-2, perhaps via the induction of **Bax**, since p53 has been shown to induce *bax* gene transcription via p53 response elements within the *bax* promoter (Miyashita et al., 1995). **Moreover, p53 levels rapidly rise following SM treatment.**

We are therefore examining the expression and phosphorylation of Bcl-2, Bcl-XL, Bad and Bax following **SM** treatment, initially by Western analysis (for immunoblotting methods, please refer to **section 2.2** above). Any alterations in these proteins (as seen in the Bcl-2 protein, below) will also be further examined at the transcriptional level by PCR to see if they are a primary event in the repression/derepression of apoptosis. Since these proteins regulate mitochondrial function and stability, **they may be important with regards to both apoptotic and necrotic responses to SM.**

2.4 Discussion and Future Course

We have demonstrated a molecular ordering of the pathway that is similar to that mediated by Fas and is also calmodulin-dependent. We have previously used a similar strategy to determine the role of sphingolipids in apoptosis (Cuvillier et al., 1998), and determined that ceramide and sphingosine-1-phosphate act downstream of caspase-8 but upstream of caspase-3. In contrast, **SM** appears to induce apoptosis upstream of caspase-8.

Future course for keratin K1 cleavage

An interesting an unexpected finding was the discovery of the potential cleavage of keratin K1 by caspases, following treatment of keratinocytes with **SM**. Thus there are convergent pathways for apoptosis and terminal differentiation in keratinocytes.

Pulse-labeling. Transfected cells are cultured in cysteine- and methionine-free DMEM for 12 h and then switched to the same medium containing 0.2 mCi/ml ^{35}S-methionine plus ^{35}S-cysteine ("EXPRE^{35}S^{35}S" system, New England Nuclear) for 1 h as described previously (Rosenthal et al., 1994). Cells are then be switched to normal medium with cold methionine (15 mg/ml) and cysteine (pulse-chase) for 12 h, and then treated with **SM** for different amounts of time.

Immunoprecipitation will be performed with the polyclonal antibody to K1 (AF87; Babco), according to procedures I have published previously (Rosenthal et al., 1994). Briefly, equal amounts of cell extracts (10 μg) are precleared overnight at 4°C with 200 μl of EBC buffer [50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5%

NP-40, and aprotinin (0.1 TIU/ml)] and 10 µl of protein A-Sepharose beads (Pharmacia). After centrifugation, the supernatants were then incubated for 1 h with 0.5 ml of NET-N buffer [20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40] containing the anti-K1 pAb (2 µg/ml). The samples were then incubated for an additional 20 min with 20 µl of a 1:1 suspension of protein A-Sepharose beads in Tris-buffered saline containing 10% bovine serum albumin. The beads are washed five times with NET-N buffer, and the proteins bound to the beads were then separated by SDS-PAGE. gels, dried, and subject to fluorography. If label appears initially in the 67 kDa K1 band, and then appears in the smaller molecular weight band over time, this indicates that the cleavage product arises from proteolysis of K1. Inhibition by VEID-CHO will indicate that this occurs at the VEID caspase-6 recognition site.

3 CHARACTERIZATION OF CHANGES IN CALMODULIN AND FAS/TNF-RELATED PROTEINS DURING SM-INDUCED APOPTOSIS, AND DETERMINATION OF THEIR IMPORTANCE AS POTENTIAL MODULATORS OF SM TOXICITY

3.1 Introduction

Recently, some compounds have been found to exert their influence via the TNF and Fas receptors, and some agents that induce apoptosis have been found to elevate the levels of the ligands for TNF or Fas or their receptors. We have obtained preliminary evidence that **SM** up-regulates both Fas and Fas L in primary human epidermal keratinocytes. In a previous study, it was determined that ultraviolet radiation, an agent that induces apoptosis in keratinocytes, up-regulates the levels of TNF α, and that partial protection of keratinocytes can be obtained by incubating cultures of keratinocytes with antibody that neutralizes TNF (Schwarz et al., 1995). Furthermore, another study showed that approximately 5% of keratinocytes express the Fas receptor, and are susceptible to Fas-mediated apoptosis. Following treatment with interferon-gamma, 32% of the keratinocytes express Fas receptor and this corresponds with the fraction of cells susceptible to Fas-mediated apoptosis (Takahashi et al., 1995). When we examined the expression of Fas receptor in primary keratinocytes by Western analysis, the levels were negligible, consistent with previous results. However, following treatment with **SM**, there was an up-regulation in the levels of both Fas receptor and Fas ligand. We also have seen activation of markers of apoptosis that are consistent with a Fas ligand-receptor, including caspase-8, caspase-3, and PARP cleavage (**see Figure above**). Thus, we are using antibodies specific to Fas and TNF receptors to both establish the role of the receptors in **SM** vesication as well as to block its apoptotic effects.

3.2 Materials and Methods

(1) ***Development of plasmids capable of introducing FADD-DN into primary and immortalized keratinocytes for grafting: Cloning, transfection, and viral infection protocols.*** Our immunoblot analysis showed that Fas and Fas L are up-regulated following SM treatment (above). We thus obtained cDNA for FADD-DN (Vishva Dixit; Genentech) ligated into the **PCDNA3 vector** (Invitrogen). Since this vector contains a strong promoter (**HCMV**), we used it to express FADD-DN in NcoI cells, which form a normal epidermis in the graft system. This construct was then transfected into Nco I cells by lipofection (lipofectamine plus; Life Technologies), and we obtained 20 G418-resistant colonies. The individual colonies, as well as pooled colonies were screened by Western analysis for the expression of FADD-DN, and three clones appear to synthesize greater quantities of the FADD-DN protein than endogenous FADD itself **(see figure below)**.

Co-immunoprecipitation of FADD and Fas/TNFR. The conceptual framework for the coimmunoprecipitation involves exploiting the importance of the recruitment of FADD to the cell surface complex in order to initiate signaling. Since the FADD protein is common to three different families of apoptosis-signaling receptors and ligands, we are performing immunoprecipitation with FADD and then look for which receptor is co-immunoprecipitated. Thus, in order to establish the importance of the up-regulation of these receptors, we will immunoprecipitate FADD protein and then perform Western analysis utilizing anti-Fas, TRNFR, or DR3 antibodies. Equal amounts of cell extracts (10 µg) are pre-cleared overnight at 4°C with 200 µl of EBC buffer [50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5% NP-40, and aprotinin (0.1 TIU/ml)] and 10 µl of protein A-Sepharose beads (Pharmacia). After centrifugation, the supernatants are then incubated for 1 h with 0.5 ml of NET-N buffer [20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40] containing the anti-FADD Ab (2 µg/ml). The samples are then incubated for an additional 20 min with 20 µl of a 1:1 suspension of protein A-Sepharose beads in Tris-buffered saline containing 10% bovine serum albumin. The beads are washed

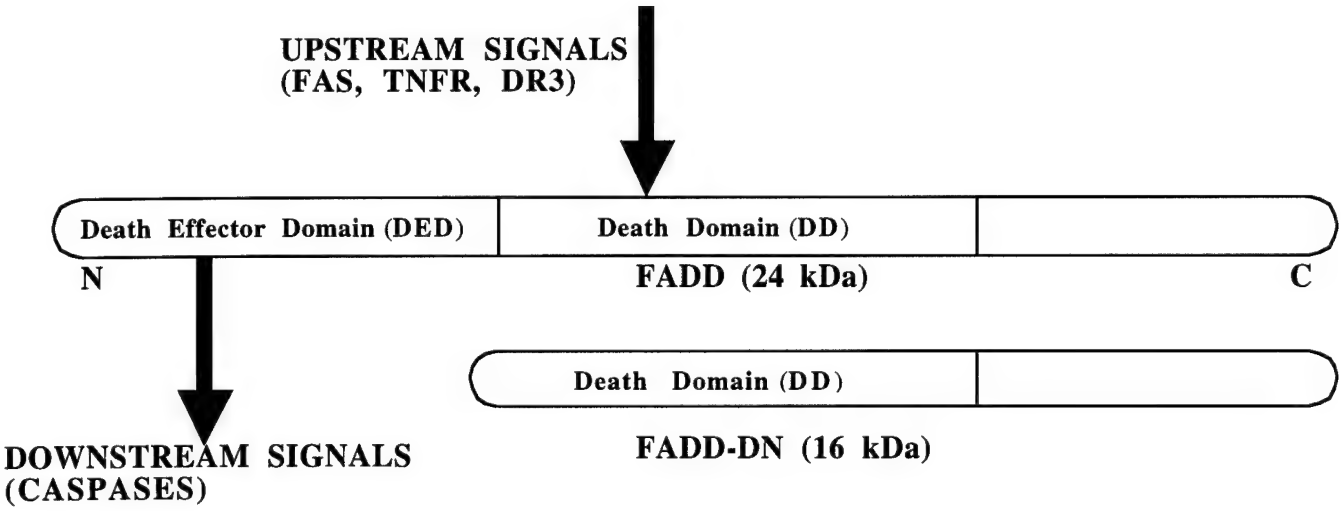
five times with NET-N buffer, and the proteins bound to the beads were then separated by SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblot analysis with the Ab to Fas or TNFR. As a positive control, we will use keratinocytes stimulated with Fas or TNF, where a complex is formed between FADD and either the Fas or TNF receptors respectively (see first figure).

In addition, members of the Fas/TNF family and their receptors may be induced at the level of transcription following stimulation by apoptosis-inducing agents, such as doxorubicin (Friesen et al., 1996; Herr et al., 1997), **and p53 has been shown to play a role in the up-regulation of Fas receptor** (Owen-Schaub et al., 1995). **Importantly, we have shown that p53 is also rapidly upregulated in keratinocytes following SM treatment, and that p53 may play a role in SM-induced apoptosis** (Stöppler et al., 1998). Thus we will examine the expression of Fas/TNF and their receptors following **SM** exposure, using **RT-PCR** as described above.

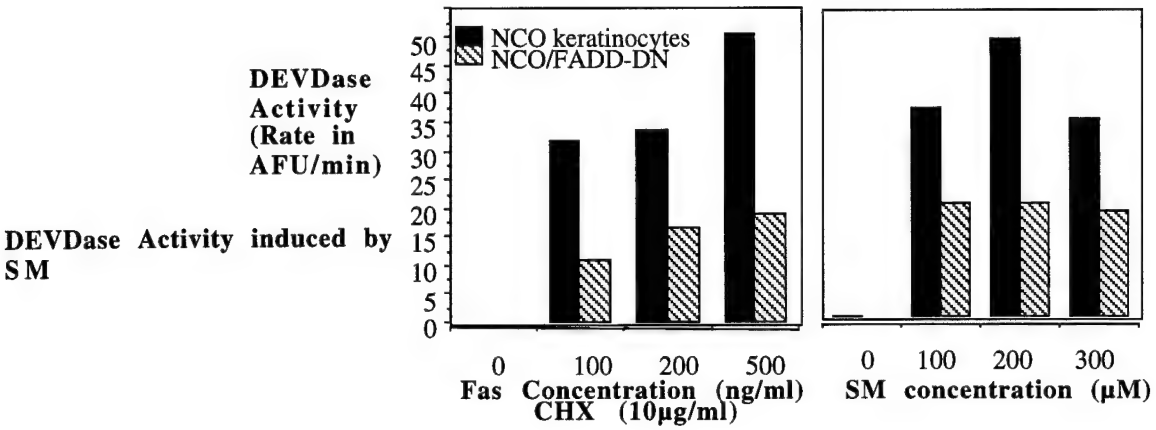
3.3 Results.

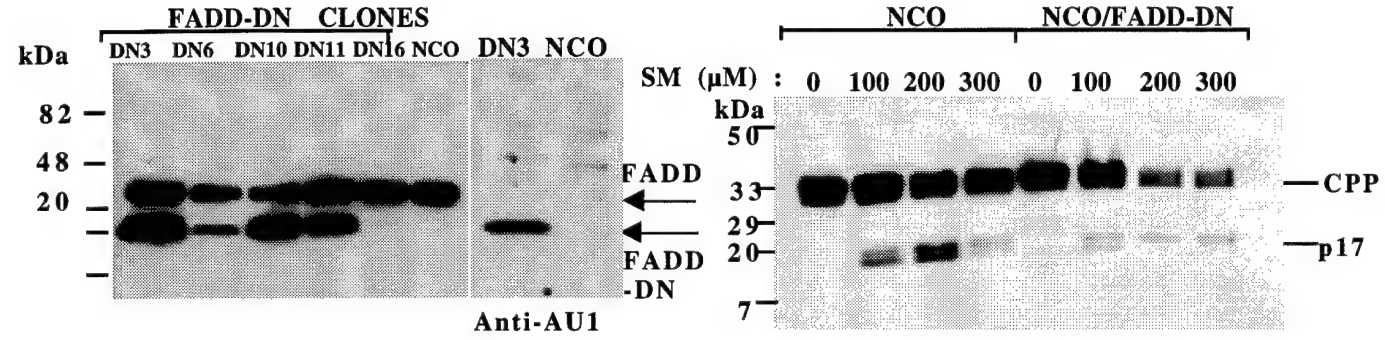
Effects of modulating the Fas-activated "death domain" (FADD) necessary for the transduction of the apoptotic signal by utilizing a dominant-negative approach (FADD-DN) and by neutralizing antibodies directed against Fas/TNF ligands and receptors.

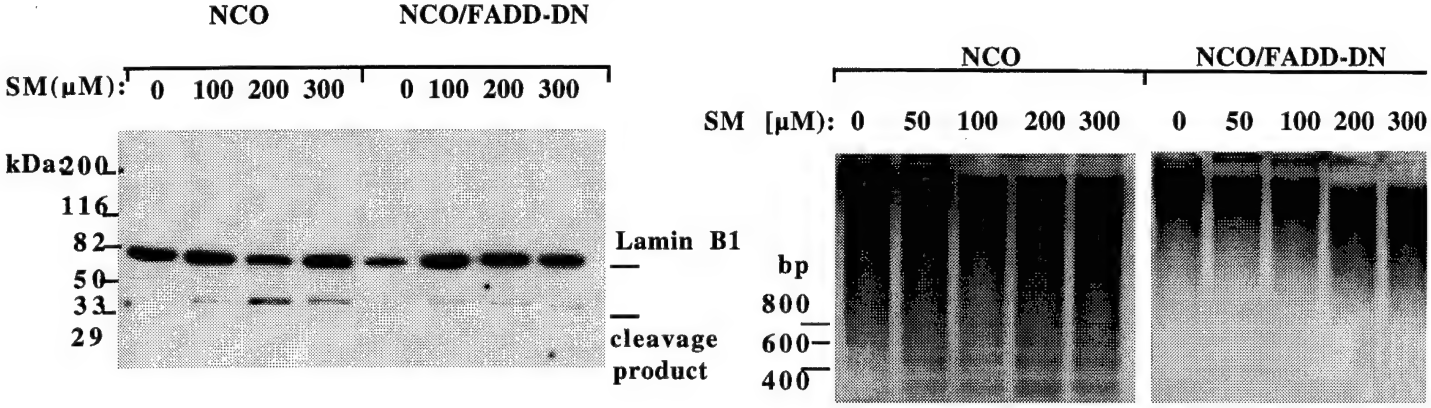
As discussed in the Introduction, many forms of apoptosis are mediated via the Fas/TNF receptor family. Upon trimeraization of the receptors, FADD is recruited to the complex via its N-terminal death domain. FADD has no intrinsic catalytic activity on its own. Its main function appears to be as an adaptor molecule that recruits upstream caspases (esp. caspase-8) to the complex via the death effector domain (DED; see figure above). We have utilized a construct that expresses a dominant-negative FADD that is missing the DED. Thus the DN FADD competes for the endogenous binding site on the receptor but cannot transmit the necessary signals downstream to caspase-8, and apoptosis that is mediated via the Fas/TNF receptor family is blocked **(see figure below)**. This system is a powerful tool to test the mechanism by which **SM** induces apoptosis.



Our results utilizing this protein are very promising. We stably transfected NcoI cells with the FADD-DN in pcDNA3, which contains the strong HCMV promoter (obtained from V. Dixit, Genentech). The first figure shows that FADD-DN is expressed in high levels in NcoI keratinocytes. The construct also contains the amino acids for the epitope AU1 which allows the protein to be tracked. We then examined the response of the FADD-DN NcoI cells to respond to either Fas or to **SM**. The figure below shows that both Fas- and **SM**-mediated apoptosis are inhibited, as measured by caspase-3 activation, lamin B1 cleavage, and DNA laddering.







3.4 Discussion

Since FADD-DN experiments appear to block markers of **SM**-induced apoptosis , further inhibition experiments are being performed utilizing neutralizing antibodies for Fas/TNF family and their receptors. Several commercial antibodies are available for this purpose. A useful antibody in this regard is the anti-human Fas antibody clone ZB4 available from Upstate Biotechnology. This more applied approach will form the basis of studies that will attempt to inhibit *in vivo* **SM**-induced apoptosis as outlinedbelow.

4 MODULATION OF PRO-APOPTOTIC PROTEINS IN SM-EXPOSED GRAFTED AND TRANSGENIC ANIMALS TO CORRELATE RELATIONSHIP BETWEEN DIFFERENTIATION, APOPTOSIS, AND VESICATION, IN ORDER TO ESTABLISH POTENTIAL THERAPEUTIC STRATEGIES.

4.1 Introduction

The model system described above has been designed to test the effects of inhibitors of calmodulin and Fas/TNF receptor family on differentiation and apoptosis in cultured human epidermal keratinocytes. We are now utilizing gene-specific inhibitors of calmodulin and FADD in the grafted epidermis, as well as in transgenic animals. Besides establishing the roles of calmodulin and FADD, there exists the possibility that similar technology can be applied to therapeutic treatment of **SM** lesions. Numerous studies have demonstrated that antisense oligonucleotides or derivatives can be applied to cultured cells, whereupon they are taken up and inhibit endogenous mRNA. Recently, several investigators have been successful in utilizing antisense oligonucleotides to specifically lower the levels of gene products in keratinocyte culture, and even in intact animals. The advantage of such technology applied to the skin is that topical application can localize treatment (**Task 9.3**). Transgenic mice, obtained by experimental integration of foreign genetic material into germ-line DNA, also provide an excellent system for studying gene regulation and function. These would include either transgenic mouse strains containing antisense to CAM or expressing FADD-DN. In these studies, newborn animals would be used to test topical mustard-induced microblistering and the effects of reduced CAM or Fas/TNF signaling on such a biological end point based on the observations of Papirmeister and McAdams (McAdams, 1956). The use of molecular biology to study **SM** pathology, *in the context of the whole animal* genetically manipulated to be altered in the proposed pathways of Ca^{2+} metabolism and Fas/TNF signaling for **SM** pathology, offers the potential for a better understanding of the mechanism of this damage for human personnel.

4.2 Materials and Methods

Grafting the engineered primary and immortalized keratinocytes, and testing for antisense and sense calmodulin expression.

keratinocytes. The two types of cells to be utilized in the grafting experiments- NHEK and NcoI have been extensively studied. For the introduction of DNA in a plasmid, a human keratinocyte cell line which could grow for long periods of time in tissue culture is required to perform the transfection experiments. For these purposes, we obtained the human keratinocytes originally generated in Dr. Richard Schlegel's laboratory at Georgetown University by immortalization of primary human keratinocytes with the appropriate regions of the HPV 16, HPV 18, or SV 40. We grafted these parent keratinocyte lines onto nude mice. Some of the lines formed carcinomas, some of the lines formed nests of cells under the host skin, and one of the lines formed no skin at all. Fortunately, the NcoI line, containing the HPV E6 and E7 genes formed a histologically normal skin layer when these cells are grafted onto nude mice. The following features are important as they relate to its normal histological appearance and its usefulness for the experiments outlined for year 3 of the contract: 1) The grafted and normal skin shows a well-defined basement membrane as well as a distinct basal cell layer, and 2) normal markers of morphological differentiation are observed, including spinous layers, granular layers, and anucleate cornified layers (see **figure page 25**).

Grafting protocol. Grafting protocols are performed by the PI at ROW labs, Gaithersburg, MD. Athymic mice have a 1 cm diameter piece of skin removed from their dorsal surface. A pellet of cells containing 8×10^6 fibroblasts + 5×10^6 keratinocytes (NHEK or NcoI) are then pipetted on top of the muscular layer within a silicon dome to protect the cells during development. The dome is removed after 1 week, and **SM** exposures are performed by Dana Anderson, USAMRICD, 6-8 weeks after grafting.

Assays for the presence of calmodulin. Using the same techniques and a similar conceptual approach that was used for PARP antisense, I have established stable lines of CAM--AS keratinocytes, and then test them for the ability to inducibly and selectively lower levels of calmodulin. Endogenous levels of CAM RNA protein and enzymatic activity were measured. As in the case of PARP, approximately 50 transfected keratinocyte clones were chosen and analyzed for correct integration of the construct using the polymerase chain reaction method. The primers that we chose for PCR analysis included a sequence homologous to the 5' UTR while the other primer are homologous to the 3' UTR CAM I sequence. In previous experiments using the same cells and the same vector to express antisense to PARP, a number of human keratinocyte clones found to contain the correct integration of the antisense construct were derived and chosen for experiments described below. PARP activity was then assayed *in vitro* at 0 time and every 24 hours thereafter. In control cells, which were

transfected with the MMTV vector alone, there was a high level of polymerase activity, which showed a slight decrease by 48 hours. In contrast, in both of the antisense clones, a significant drop in polymerase activity was observed. By Western analysis, the amount of PARP protein is significantly reduced upon induction of the antisense human keratinocyte clones, whereas, there is no significant change in polymerase protein levels in control cells.

***In situ* hybridization** *In situ* hybridization was performed as we described previously (Rosenthal et al., 1995). Frozen skin sections are heated at 50° for 1 min, then allowed to dry for 1 h at room temperature. Slides are then fixed in 4% paraformaldehyde, and dehydrated in increasing concentrations of ethanol. Cells are permeabilized by incubation with 5 mM $MgCl_2$ followed by 1M glycine, each for 10 min, then post-fixed in 4% paraformaldehyde. Cells are rinsed in PBS, and dehydrated in ethanol. Pre-hybridization and hybridization is performed with ^{35}S-labeled riboprobes at 10^7 dpm/ml as described in Methods in Enzymology (Shivers et al., 1986). After incubation with RNase A (50 µg/ml) + T1 (500 U/ml), and SSC washes, slides are immersed in NTB-2 emulsion (Kodak) and developed after 1 to 3 weeks.

Utilizing this technique, we can detect the levels of both endogenous sense and antisense calmodulin in the graft. Taken together, these data provide the basis for work that utilize antisense constructs to reduce the levels of CAM and CAM-related proteins, and correspondingly lower their activity in the human keratinocytes grafted to mice. I have already used the *in situ* hybridization technique to detect PARP antisense RNA expression in skin grafts (Rosenthal et al., 1995); this same technique was employed to detect CAM antisense RNA. The graft would be subsequently treated with **SM** (vapor or topically) and tested for changes in differentiation and apoptosis-related marker expression, as well as reduction of **SM** pathology.

4.3 Results

(1) *Reducing levels of Calmodulin in skin grafts*

Development of plasmids and retroviral vectors capable of introducing CAM I antisense RNA into primary and immortalized keratinocytes for grafting: Cloning, transfection, and viral infection protocols. Our RNA expression studies showed that calmodulin I is the main CAM RNA species expressed in keratinocytes. We thus derived the cDNA for CAM I ourselves, utilizing RT-PCR of mRNA derived from NHEK, and specific primers, as described. This 900 bp CAM I fragment was then ligated into the **PCR3.1 vector** (Invitrogen) in the sense and antisense orientation. Since this vector contains a strong promoter (**HCMV**), we used it to express CAM I antisense RNA in NcoI cells, which form a normal epidermis in the graft system. The antisense construct was then transfected into Nco I cells by lipofection (lipofectamine plus; Life Technologies), and we obtained 15 G418-resistant colonies. The individual colonies, as well as pooled colonies were screened by Western analysis for the expression of CAM I, and two clones appear to synthesize little or no endogenous calmodulin. These NcoI cells, along with control cells transfected with empty vector alone are now being grafted, since they have been demonstrated to form a normal skin previously (Rosenthal et al., 1995).

The pCR3.1CAM I clone was then digested to remove the CAM I insert, which was then ligated the antisense orientation into the **retroviral vector, LXSN** (Clontech). In addition to CAM I, we have cloned the other two human calmodulin cDNAs (obtained from Dr. Emmanuel Strehler; Mayo clinic) into the LXSN vector; the CAM II and III constructs are shown in the **figure below**. We have used the LXSN vector previously to introduce E6 and E7 genes into primary keratinocytes (Stöppler et al., 1998).

Recently, we have obtained a new retrovirus packaging cell line that we are utilizing to derived high-titer ($>10^6$/ml) retrovirus following transient transfection (Phoenix packaging line; Dr. Gary Nolan; Stanford), eliminating our earlier need to screen clones of virus-producing cells (a time-consuming process). Thus, we have already derived high-titer viral supernatants from LXSN CAM I antisense constructs, and have begun to infect Nco I cells in addition to using the p3.1 vector. Our initial results indicate a strong reduction in the endogenous levels of calmodulin.

As in our previous studies, these packaged retroviruses are being used to infect NHEK cells and then grafted onto nude mice. Although viral titer is high, we are performing a selection in G418 to ensure that 100% of the cells are expressing CAM antisense RNA. We used the same conditions as in our previous study (Stöppler et al., 1998), in which NHEK were infected with the derivatives of the amphotrophic LXSN retrovirus expressing the various HPV-16 open reading frames (E6, E7, or E6 plus E7). Retrovirus-infected cells were then selected in G418 (100 µg/ml medium) for 10 days.

(2) *Modulation of the Fas-FADD pathway response in SM exposed keratinocytes in reconstituted skin grafted onto nude mice.* We have begun to utilize the graft system to generate a human epidermis containing the FADD-DN3 clones (shown above). We have very preliminary results utilizing 4 animals grafted with NcoI (control), and 4 animals grafted with the DN3 clone of NcoI. These studies were performed in collaboration with Dana Anderson and Dr. Larry Micheltree of USAMRICD. Additional animals are being grafted to obtain significant numbers for histology. We presently have frozen and fixed sections derived from these animals which we are analyzing for the expression of FADD-DN using the AU1 antibody, which recognizes the specific AU1 epitope placed on the FADD-DN protein. In addition, we are examining markers of in vivo apoptosis and differentiation using the techniques described above. Any changes observed at the protein level are being further analyzed utilizing *in situ* hybridization described previously (Rosenthal et al., 1995).

Animal	Site	Exposure Time (min)	Pustular Epidermitis	Epidermal Necrosis	Microvesicle (Cleft)	Follicular Involvement
FADD-DN 1	**GRAFT**	6	0	0	**0**	0
FADD-DN 2	"	6	2	2	**1**	0
FADD-DN 3	"	8	0	0	**0**	0
FADD-DN 4	"	8	0	1	**0**	0
CONTROL1	"	6	0	0	**0**	0
CONTROL2	"	6	0	0	**0**	0
CONTROL3	"	8	0	0	**2**	0
CONTROL4	"	8	2	3	**3**	0
FADD-DN 1	**HOST**	6	2	4	2	3
FADD-DN 2	"	6	2	3	1	3
FADD-DN 3	"	8	1	2	0	2
FADD-DN 4	"	8	1	4	3	3
CONTROL1	"	6	2	4	2	4
CONTROL2	"	6	0	1	0	1
CONTROL3	"	8	4	1	2	3
CONTROL4	"	8	2	4	3	2

(3) *Establishing transgenic animals with K1-antisense calmodulin, or K1-FADD-DN*

a. *A skin-specific vector.* While working for several years in the Laboratory of Cellular Carcinogenesis and Tumor Promotion at NIH under Dr. Stuart Yuspa, an acknowledged authority on the molecular biology of skin, I was able to define DNA regulatory elements that mediate response of the keratin 1 (K1) gene (Huff et al., 1993; Rosenthal et al., 1991). Accordingly, I have cloned regions spanning the 5' and 3' flanking sequences, coding regions and introns from the human keratin 1 gene for the expression of calmodulin antisense RNA, specifically in keratinocytes. As we showed previously (Huff et al., 1993; Rosenthal et al., 1991), each of the HK1 enhancer elements appears to have a distinct function. For example, the 422-bp proximal element seems to mediate induction by Ca^{2+}. In contrast, the distal 158-bp element is involved in determining the absolute level of expression of keratin (or a foreign gene inserted near it) in skin cells since partial or complete deletion was shown to cause a reduction in activity but not of Ca^{2+} inducibility.

This approach takes advantage of skin-specific expression of CAM and FADD-DN constructs to examine their roles in response to **SM** in transgenic mice. Human CAM and FADD-DN sequences have therefore been inserted into the K1 vector, in order to drive the epidermal-specific expression of CAM antisense or FADD-DN in transgenic mice.

b. *Methods for Transgenic Mice.* In brief, the plasmid are being injected into the pronucleus of oocytes; subsequently the oocytes are reintroduced into mock-fertilized female mice. After birth and weaning (approximately 6 weeks total time), DNA derived from the tails of these "founder" mice will be tested for the integration of the foreign new sequences into total tissue cellular DNA by well-established PCR and Southern hybridization techniques

4.4 Discussion

Testing of CAM and FADD constructs in the graft system to determine if the response is altered following SM treatment: In vivo markers for differentiation, apoptosis and vesication

(1) *Detection of differentiation markers.* Utilizing NcoI cells in our system, we have previously demonstrated the correct expression of keratins K1, K10 and K14 within the grafted epidermis (Rosenthal et al., 1995). These differentiation markers are therefore detected employing the technique that we have previously described for this system (Rosenthal et al., 1995), and similar to that described below, with the exception that TCA and ethanol fixation are not used to detect the epidermal keratins in frozen sections.

(2) *Detection of earliest stages of apoptosis.* The earliest stage of apoptosis is marked by the activation of PARP (Simbulan-Rosenthal et al., 1998). Thus, we are assaying for the presence of polymer using indirect immunofluorescence of sections as described previously (Rosenthal et al., 1997). Frozen skin sections are fixed with 10% (w/v) ice-cold trichloroacetic acid for 10 min, and dehydrated in 70%, 90%, and absolute ethanol for 3 min each at -20°C. The slides are then incubated overnight in a humid chamber at room temperature with antibodies to PAR (1:250 dilution) in phosphate-buffered saline (PBS) containing 12% bovine serum albumin (BSA). After washing with PBS, sections are incubated for 1 h with biotinylated anti-mouse IgG (1:400 dilution in PBS-BSA), washed, and incubated for 30 min with streptavidin-conjugated Texas red (1:800 dilution in PBS-BSA). Cells are finally mounted with PBS containing 80% glycerol and observed with a Zeiss fluorescence microscope. All exposure times are identical to allow comparisons of relative staining intensities at various times during apoptosis.

(3) *Detection of mid-stages of apoptosis*: active caspases and their substrate cleavage products. To observe activation of caspases in skin sections, we are exploiting the properties of several antibodies that recognize only the cleavage products of caspase- 7, caspase-8 and PARP, but do not recognize the full-length proteins. The caspase antibodies are available from Dr. Edward Gelmann (Georgetown University) while the antibody against the 24 kDa cleavage product of PARP has been developed by our laboratory in collaboration with Dr. Intisar Hussein (Glaxo).
Antibodies to PARP-DBD are derived by immunization of rabbits with a peptide corresponding to amino acids 25 to 41 of human PARP. The specificity of this antibody is demonstrated above, and its use for the detection of apoptotic cells has been described previously (Rosenthal et al., 1997).

(4) *Detection of late stage of apoptosis: DNA fragmentation.* DNA breaks are detected *in situ* using a Klenow fragment-based assay system (TACS1; Trevigen). Cells are fixed and labeled with biotinylated nucleotides, using streptavidin-conjugated horseradish peroxidase and diamino-benzidine for detection. Cells are counter-stained with methyl green. Brown nuclei are positive for Klenow labeling. DNA nucleosome ladders are observed by isolation of total genomic DNA and agarose gel electrophoresis as described previously (Nicholson et al., 1995).

(5) *Detection of vesication.* The probability of success in utilizing an end point of micro or macro blisters for mustard applications in such engineered human skin regions has been strengthened by our owen observations made in experiments performed in collaboration with Dana Anderson and Dr. Larry Micheltree of USAMRICD, as well as those of Papirmeister and colleagues, that human skin grafts transplanted onto nude mice have been successful to examine **SM**-induced NAD depletion (Gross et al., 1988; Meier et al., 1984; Papirmeister et al., 1991; Petrali et al., 1990; Smith et al., 1990; Smith et al., 1991; Smulson, 1990). The model has also been used to assess the protection afforded against **SM**-induced NAD depletion by the systematically administered PADPRP inhibitors 3-aminobenzamide and 3-methoxybenzamide. Microblisters in **SM**-exposed skin grafts on nude mice have also been verified by van Genderen and colleagues (van Genderen and Wolthuis, 1986).

5. CONCLUSIONS

- **SM** induces markers of apoptosis in primary (NHEK) and immortalized keratinocytes.
- Activation of caspases is calmodulin and Fas/TNF-dependent.
- Molecular ordering of specific caspase activation suggests that **SM** operates via a Fas/TNF pathway.
- Fas and Fas L are up-regulated in keratinocytes exposed to **SM**.
- Calmodulin antisense vectors have been constructed and have been shown to lower endogenous levels of CAM in immortalized human keratinocytes (NcoI).
- Calmodulin antisense and sense retroviruses have been constructed for the infection of primary human keratinocytes.
- Grafted human keratinocytes expressing FADD-DN show a reduced response to **SM** *in vivo*.

Plans/Milestones for the Next Quarter

- Grafting of calmodulin antisense keratinocytes.

6. ACCOMPLISHMENT OF TASKS

During the past year, we have been successful in accomplishing the majority of the remaining tasks. **Task 7** (C 2.5.1) has been accomplished. **Task 7** (C2.5.2) has been initiated. **Task 8** has been initiated. **Task 9** (C.2.7.1) has been accomplished for the FADD-DN clones and is being performed for the calmodulin antisense as well. **Task 9** (C2.7 (2)) has been accomplished for FADD-DN clones. **Task 10** is in preparation.

7. REFERENCES

Alnemri, E., Livingston, D., Nicholson, D., Salvesen, G., Thornberry, N., Wong, W., and Yuan, J. (1996). Human ICE/CED-3 protease nomenclature [letter]. Cell *87*, 171.

Cuvillier, O., Rosenthal, D. S., Smulson, M. E., and Spiegel, S. (1998). Sphingosine 1-phosphate inhibits activation of caspases that cleave poly(ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes. J Biol Chem *273*, 2910-6.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231-41.

del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. (1997). Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science *278*, 687-9.

Friesen, C., Herr, I., Krammer, P. H., and Debatin, K. M. (1996). Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nat Med *2*, 574-7.

Gross, C. L., Innace, J. K., Smith, W. J., Krebs, R. C., and Meier, H. L. (1988). Alteration of lymphocyte glutathione levels affects sulfur mustard cytotoxicity. In Proceedings of the meeting of NATO Research Study Group, Panel VIII/RSG-3 (Washington, D.C.

Herr, I., Wilhelm, D., Bohler, T., Angel, P., and Debatin, K. M. (1997). Activation of CD95 (APO-1/Fas) signaling by ceramide mediates cancer therapy-induced apoptosis. Embo J *16*, 6200-8.

Hsu, S. Y., Kaipia, A., Zhu, L., and Hsueh, A. J. (1997). Interference of BAD (Bcl-xL/Bcl-2-associated death promoter)-induced apoptosis in mammalian cells by 14-3-3 isoforms and P11. Mol Endocrinol *11*, 1858-67.

Huff, C. A., Yuspa, S. H., and Rosenthal, D. (1993). Identification of control elements 3' to the human keratin 1 gene that regulate cell type and differentiation-specific expression. J Biol Chem *268*, 377-84.

McAdams, A. J., Jr. (1956). A study of mustard vesication. J. Invest. Dermatol. *26*, 317-326.

McLean, W. H., Eady, R. A., Dopping-Hepenstal, P. J., McMillan, J. R., Leigh, I. M., Navsaria, H. A., Higgins, C., Harper, J. I., Paige, D. G., Morley, S. M., and et al. (1994). Mutations in the rod 1A domain of keratins 1 and 10 in bullous congenital ichthyosiform erythroderma (BCIE). J Invest Dermatol *102*, 24-30.

Meier, H. L., Gross, C. L., Papirmeister, B., and Daszkiewicz, J. E. (1984). The use of human leukocytes as a model for studying the biochemical effects of chemical warfare (CW) agents. In Proceedings of the Fourth Annual Chemical Defense Bioscience Review (Aberdeen Proving Ground, Maryland.

Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T. T., Yu, V. L., and Miller, D. K. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature *376*, 37-43.

Owen-Schaub, L. B., Zhang, W., Cusack, J. C., Angelo, L. S., Santee, S. M., Fujiwara, T., Roth, J. A., Deisseroth, A. B., Zhang, W. W., Kruzel, E., and et al. (1995). Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. Mol Cell Biol *15*, 3032-40.

Papirmeister, B., Feister, A. J., Robinson, S. I., and Ford, R. D. (1991). Medical defense against mustard gas: toxic mechanisms and pharmacological implications, 1st Edition (Boca Raton: CRC Press).

Petrali, J. P., Oglesby, S. B., and Mills, K. R. (1990). Ultrastructural correlates of sulfur mustard toxicity. J. Toxicol. Cut. Ocul. Toxicol. *9*, 193-214.

Rosenthal, D., Hong, T., Cherney, B., Zhang, S., Shima, T., Danielsen, M., and Smulson, M. (1994). Expression and characterization of a fusion protein containing the DNA-binding domain of the glucocorticoid receptor and the catalytic domain of poly(ADP-ribose) polymerase. Bioch. Biophys. Res. Commun. *202*, 880-887.

Rosenthal, D. S., Chung, S., Steinert, P. M., Huff, C. A., Yuspa, S. H., and Roop, D. R. (1991). The human K1 gene is regulated independently by calcium and retinoids in transgenic mouse keratinocytes. Cell Growth and Differentiation *2*, 107-113.

Rosenthal, D. S., Ding, R., Simbulan-Rosenthal, C. M. G., Vaillancourt, J. P., Nicholson, D. W., and Smulson, M. E. (1997). Intact cell evidence fo the early snthesis, and subsequent late apopain-mediated suppression, of poly(ADP-ribose) during apoptosis. Exp. Cell Res. *232*, 313-321.

Rosenthal, D. S., Rosenthal, C. M. G., Iyer, S., Smith, W., Ray, R., and Smulson, M. E. (1998).
Sulfur mustard induces terminal differentiation and apoptosis in keratinocytes via a Ca2+-calmodulin and apopain-dependent pathway. J Invest Dermatol *111*, 64-71.

Rosenthal, D. S., Shima, T. B., Celli, G., De Luca, L. M., and Smulson, M. E. (1995). An engineered human skin model using poly(ADP-ribose) polymerase antisense expression shows a reduced response to DNA damage. J. Invest. Dermatol. *105*, 38-44.

Scheid, M. P., and Duronio, V. (1998). Dissociation of cytokine-induced phosphorylation of Bad and activation of PKB/akt: involvement of MEK upstream of Bad phosphorylation. Proc Natl Acad Sci U S A *95*, 7439-44.

Schlegel, R., Phelps, W. C., Zhang, Y. L., and Barbosa, M. (1988). Quantitative keratinocyte assay detects two biological activities of human papillomavirus DNA and identifies viral types associated with cervical carcinoma. Embo J *7*, 3181-7.

Schwarz, A., Bhardwaj, R., Aragane, Y., Mahnke, K., Riemann, H., Metze, D., Luger, T., and Schwarz, T. (1995). Ultraviolet-B-induced apoptosis of keratinocytes: evidence for partial involvement of tumor necrosis factor-alpha in the formation of sunburn cells. J Invest Dermatol *104*, 922-7.

Shibasaki, F., Kondo, E., Akagi, T., and McKeon, F. (1997). Suppression of signalling through transcription factor NF-AT by interactions between calcineurin and Bcl-2. Nature *386*, 728-31.

Shivers, B. D., Schachter, B. S., and Pfaff, D. W. (1986). In situ hybridization for the study of gene expression in the brain. Methods Enzymol *124*, 497-510.

Simbulan-Rosenthal, C. M., Rosenthal, D. S., Iyer, S., Boulares, A. H., and Smulson, M. E. (1998). Transient poly(ADP-ribosyl)ation of nuclear proteins and role for poly(ADP-ribose) polymerase in the early stages of apoptosis. J. Biol. Chem. *273*, 13703-13712.

Smith, W. J., Gross, C. L., Chan, P., and Meier, H. L. (1990). The use of human epidermal keratinocytes in culture as a model for studying the biochemical mechanisms of sulfur mustard toxicity. Cell Biol. Toxicol. *6*, 285-91.

Smith, W. J., Sanders, K. M., Gales, Y. A., and Gross, C. L. (1991). Flow cytometric analysis of toxicity by vesicating agents in human cells *in vitro*. Toxicol. *10*.

Smulson, M. E. (1990). Molecular biology basis for the response of poly(ADP-rib) polymerase and NAD metabolism to DNA damage caused by mustard alkylating agents: Georgetown University Medical School).

Stöppler, H., Stöppler, M. C., Johnson, E., Simbulan-Rosenthal, C. M., Smulson, M. E., Iyer, S., Rosenthal, D. S., and Schlegel, R. (1998). The E7 protein of human papillomavirus type 16 sensitizes primary human keratinocytes to apoptosis. Oncogene *17*, 1207-1214.

Takahashi, H., Kobayashi, H., Hashimoto, Y., Matsuo, S., and Iizuka, H. (1995). Interferon-gamma-dependent stimulation of Fas antigen in SV40-transformed human keratinocytes: modulation of the apoptotic process by protein kinase C. J Invest Dermatol *105*, 810-5.

van Genderen, J., and Wolthuis, O. L. (1986). New models for testing skin toxicity. In Skin Models: Models to Study Function and Disease of Skin, R. Marks and G. Plewig, eds. (Berlin: Springer-Verlag).

Wang, H. G., Mckeon, F., and Reed, J. C. (1997). Dephosphorylation of pro-apoptotic protein Bad by calcineurin results in association with intracellular membranes. In Programmed Cell Death (Cold Spring Harbor, NY.

Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995). Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell *80*, 285-91.

Zha, J., Harada, H., Osipov, K., Jockel, J., Waksman, G., and Korsmeyer, S. J. (1997). BH3 domain of BAD is required for heterodimerization with BCL-XL and pro-apoptotic activity. J Biol Chem *272*, 24101-4.

Zundel, W., and Giaccia, A. (1998). Inhibition of the anti-apoptotic PI(3)K/Akt/Bad pathway by stress. Genes Dev *12*, 1941-6.

8. CHRONOLOGICAL BIBLIOGRAPHY AND PERSONNEL

Publications:

1. Stöppler, H., Stöppler, M. C., Johnson, E., Simbulan-Rosenthal, C. M. G., Iyer, S., Smulson, M. E., **Rosenthal, D. S.***, and Schlegel, R. The human papillomavirus 16 (HPV-16) E7 protein sensitizes primary human keratinocytes to apoptosis. *Oncogene*, **17**: 1207-1214 (1998). * **corresponding author (subject contract support is acknowledged).**

2. Simbulan-Rosenthal, C. M. G., **Rosenthal, D. S.**, Iyer, S, Yoshihara, K., and Smulson, M. E. Requirement for PARP and poly(ADP-ribosyl)ation in apoptosis and DNA replication. *Molecular and Cellular Biochemis*try, **193**:137-148 (1999).
(subject contract support is acknowledged).

3. Iyer, S., Chaplin, D., **Rosenthal, D. S.**, Boulares, A. H., Li, L., and Smulson, M. Induction of apoptosis in proliferating human endothelial cells by the tumor-specific anti-angiogenesis agent combretastatin A-4. *Cancer Research*, **58**: 4510-4514 (1998).

4. **Rosenthal, D. S.**, Rosenthal, C. M. G., Iyer, S., Smith, W., Ray, R., and Smulson, M. E. Sulfur mustard induces terminal differentiation and apoptosis in keratinocytes via a Ca^{2+}-calmodulin and apopain-dependent pathway. *J. Invest. Dermatol.* **111**(1): 64-71 (1998).
(subject contract support is acknowledged).

5. Simbulan-Rosenthal, C. M. G., **Rosenthal, D. S.**, Iyer, S., Boulares, A. H., and Smulson, M. E. Transient poly(ADP-ribosyl)ation of nuclear proteins and role of poly(ADP-ribose) polymerase in the early stages of apoptosis. *J. Biol. Chem.* **273**(22): 13703-13712 (1998).
(subject contract support is acknowledged).

6. Simbulan-Rosenthal, C. M. G., **Rosenthal, D. S.**, Boulares, A. H., Hickey, R., Malkas, L., Coll, J., and Smulson, M. E. Regulation of the expression or recruitment of components of the DNA synthesome by poly(ADP-ribose). *Biochemistry* **37**(26): 9363-9370 (1998).
(subject contract support is acknowledged).

7. Cuvillier, O., **Rosenthal, D. S.**, Smulson, M. E., and Spiegel, S. Sphingosine 1-phosphate inhibits activation of caspases that cleave poly(ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis. *J. Biol. Chem.* **273**(5): 2910-2916 (1998).

8. Smulson, M.E., Pang, D., Jung, M., Dimtchev, A., Chasovskikh, S., Spoonde, A., Simbulan-Rosenthal, C., **Rosenthal, D.**, Yakovlev, A., and Dritschilo, A. Irreversible binding of poly(ADP-ribose) polymerase cleavage product to DNA ends revealed by atomic force microscopy: possible role in apoptosis. *Cancer* Research **58**: 3495-3498 (1998).

9. **Rosenthal, D. S.**, Simbulan-Rosenthal, C. M., Iyer, S., Smith, W., Ray, R., and Smulson, M. E. Calmodulin, poly(ADP-ribose) polymerase, and p53 are targets for modulating the effects of sulfur mustard. *Proceedings of the Medical Defense Bioscience Review* (1998).
(subject contract support is acknowledged).

10. Simbulan-Rosenthal, C. M., **Rosenthal, D. S.**, Ding, R., Bhatia, K., and Smulson, M. E. Poly(ADP-ribosyl)ation of p53 during apoptosis in human osteosarcoma cells. *Bioch. Biophys. Res. Commun.*, **253**: 864-868 (1998).
(subject contract support is acknowledged).

11. **Rosenthal, D. S.**, Ding, R., Simbulan-Rosenthal, C. M. G., Cherney, B., and Vanek, P. and Smulson, M. E. Detection of DNA breaks in apoptotic cells utilizing the DNA binding domain of poly(ADP-ribose) polymerase with fluorescence microscopy. *Nucleic Acids Research* **27**(7):1437-1441 (1997).

12. **Rosenthal, D. S.**, Ding, R., Simbulan-Rosenthal, C. M. G., Vaillancourt, J. P., and Nicholson, D. W. Intact cell evidence for the early synthesis, and subsequent late apopain-mediated suppression of poly(ADP-ribose) during apoptosis. *Experimental Cell Research* **232**, 313-321 (1997).
(subject contract support is acknowledged).

13. **Rosenthal, D. S.**, and Smulson, M. E. PARP cleavage and apoptosis. *Bioradiations* **99:** 7-9 (1997)

Personnel

Dean S. Rosenthal, Ph.D.	60% effort
Wen Fang Liu, Technician	100% effort